BEST AVAILABLE IMAGES

Defective images within this document are accurate representation of The original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

09/498704 # 8

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 9/127 — A1

(11) International Publication Number: **WO 96/25147**

(43) International Publication Date: 22 August 1996 (22.08.96)

(21) International Application Number: PCT/US96/02005

(22) International Filing Date: 14 February 1996 (14.02.96)

(30) Priority Data:
08/388,374 14 February 1995 (14.02.95) US

(71) Applicant: SEQUUS PHARMACEUTICALS, INC. [US/US]; 960 Hamilton Court, Menlo Park, CA 94025 (US).

(72) Inventors: WOODLE, Martin, C.; 445 Oak Grove Avenue #3, Menlo Park, CA 94025 (US). ZALIPSKY, Samuel; 1202 Truman, Redwood City, CA 94061 (US). MARTIN, Francis, J.; 160 San Fernando Way, San Francisco, CA 94127 (US).

(74) Agent: EVANS, Susan, T.; Dehlinger & Associates, P.O. Box 60850, Palo Alto, CA 94306-1546 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: LIPOSOME COMPOSITION AND METHOD FOR ADMINISTERING LIPOSOME-LOADABLE DRUGS

(57) Abstract

A liposome composition and method of use in administering a compound having a reactive hydroxyl group to a selected *in vivo* site are disclosed. The composition includes a suspension of liposomes having an inside/outside pH gradient, and a conjugate of the compound with an adduct effective to maintain the compound in liposome-encapsulated form in response to the pH gradient.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

LIPOSOME COMPOSITION AND METHOD FOR ADMINISTERING LIPOSOME-LOADABLE DRUGS

Field of the Invention

The present invention relates to a liposome composition and method of use in administering a compound, at high drug concentration, in liposome-encapsulated form.

References

Adams, G., *Radiation Research* 132:129-139 (1992).

Aggarwal, S., *et al.*, *J. Med. Chem.* 33:1505-1510 (1990).

Allen, R.M., *et al.*, *Clin. Neuropharmacol.* 6 (Suppl. 1):S64 (1983).

Collins, P., *et al.*, *J. Antimicrob. Chemoth.* 5:431 (1979).

Cramer, J.A., and Prestegard, J.H., *Biochem. and Biophys. Res. Comm.* 75(2):295 (1977).

Deamer, D.W., *et al.*, *Biochim. et Biophys. Acta* 274:323 (1972).

DeClercq, E., *Biochem. Pharm.* 37:1789-1790 (1988).

Fischl, M.A., *et al.*, *New Eng. J. Med.*, 317(4):185 (1987).

Froehler, B., *et al.*, *Nucl. Acids Res.* 11:8031-8036. (1983).

Galbraith, A.W., *et al.*, *Lancet* 1026 (1969).

Greene, T.W., *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, 2nd ed., John Wiley & Sons, New York, NY (1991).

Harrigan, P., *et al.*, *Biochimica et Biophysica Acta* 1149:329-338 (1993).

Hassner, A., *et al.*, *Tetrahedron Lett.*, 4475 (1978).

Holmberg, K., *et al.*, *Acta Chem. Scand.* Sec. B, 33:410 (1979).

Mabrey, S., *et al.*, *Biochem.* 17:2464-2468 (1978).

Martin, F.J., In: SPECIALIZED DRUG DELIVERY SYSTEMS-MANUFACTURING AND PRODUCTION TECHNOLOGY, (P. Tyle, ed.) Marcel Dekker, New York, pp. 267-316 (1990).

Martin, J.D., Ed, NUCLEOTIDE ANALOGUES AS ANTIVIRAL AGENTS, ACS Symposium Series 401, American Chemical Society, Washington DC (1989).

McBride, L.J., *et al.*, *Tetrahedron Lett.* 24:2953-2956 (1983).

Nicholson, K.G., *Lancet II*: 617 (1984a).

Nicholson, K.G., *Lancet II*: 503 (1984b).

Saari, W., *et al.*, *J. Med. Chem.* 33:2590-2595 (1990).

Shenoy, M., *et al.*, *Cancer Investigation* 10(6):533-551 (1992).

Sidwell, R.W. in CHEMOTHERAPY OF INFECTIOUS DISEASES (H. Gadebusch, ed.) pp. 31-54, CRC Press, Cleveland.

Smith, K.O., *et al.*, *Antimicrob. Agts. Chemother.* 22:55 (1982).

Szoka, F., Jr., *et al. Ann. Rev. Biophys. Bioeng.* 9:467-508 (1980).

Tsong, T.Y., *Biochem.* 14:5409-5414, 5415-5417 (1975).

Yatvin, M.B., and Lelkes, P.I., *Med. Phys.* 9(2):149 (1982).

Background of the Invention

Liposome delivery systems have been proposed for a variety of drugs. Liposome-drug formulations offer the potential of improved drug-delivery properties, such as enhanced blood circulation time and controlled drug release.

Liposomes carrying therapeutic agents, such as anti-tumor, imaging agents and the like, have also been shown to be effective for targeting to selected tissues, such as liver, or to non-RES (reticuloendothelial system) tissue, to solid tumors, lymph, and sites of inflammation (Martin, F.J., 1990). Site-specific targeting offers the potential of reducing side-effects and increased dosing at the target site.

In preparing liposomes for therapeutic use, it is usually desirable to load high concentrations of drug into the liposomal carrier. In addition, the liposomes should be able to retain the encapsulated drug if improved therapeutic efficacy is to be realized.

Several methods for loading liposomes are known in the art. Liposomes may be passively loaded by adding the drug during hydration of vesicle-forming lipids. The efficiency of passive loading, at least in the case of water-soluble drugs, is limited by relatively poor encapsulation efficiency, usually less than about 15% of the total drug present in the hydrating medium. Passive loading also limits the internal concentrations of drug that can be achieved.

Alternatively, enhanced drug loading may be achieved by a reverse-phase evaporation method (Szoka, *et al.*, 1980) and can encapsulate up to 50% of active agent. However, this technology is not readily adaptable to commercial liposome production, because of the requirement for large volumes of volatile solvents. Additionally, liposomes produced by this technique must be sized down, *e.g.*, by extrusion, for parenteral use. Downsizing is a somewhat inefficient process which leads to further liposome losses.

Active drug loading against an ion gradient has also been proposed (Yatvin and Lelkes, 1982; Deamer, *et al.*, 1972; Cramer and Prestegard, 1977). Active drug loading can provide improved trapping efficiencies and markedly enhance drug retention properties (Harrigan, *et al.*, 1993). Heretofore, this approach has been limited to drugs with ionizable groups, typically a free amine.

Summary of the Invention

The invention includes, in one aspect, a liposomal composition for use in administering to a subject, a compound having a derivatizable hydroxyl group. The compound is one which is substantially uncharged at a neutral pH. The composition includes an external bulk-phase medium and, suspended in the medium, liposomes having an internal aqueous phase at a pH which forms an inside/outside pH gradient with respect to the internal aqueous phase and the external medium. Encapsulated within the liposomes is a compound conjugate composed of the compound to be administered and an adduct conjugated to the compound's derivatizable hydroxyl group by an ester linkage, where the adduct is effective to maintain the compound conjugate in liposome-encapsulated form in response to the pH gradient.

In one general embodiment, the inside/outside pH gradient is a lower inside/higher outside pH gradient.

Exemplary adducts for use in the present invention include, for example, amino acids. The amino acid is coupled to the compound through an ester linkage formed between the compound's hydroxyl group and the α carboxyl group of the amino acid.

The compound may be, for example, a radiosensitizer, such as a halogenated deoxyuridine, a nitroimidazole, an anthraquinone, a 2-nitrofuran, a benzoquinone, or a naphthoquinone. Alternatively, the compound may be an anti-viral nucleoside analog, such as 5'-chlorodeoxyuridine, 5'-bromodeoxyuridine, 5'-iododeoxyuridine, or 5'-fluorodeoxyuridine.

The lower inside/higher outside pH gradient may be produced by a higher inside/lower outside ammonium ion gradient, preferably where the counterion of an ammonium salt is effective to decrease the solubility of the compound conjugate in the internal aqueous phase.

For improved stability in retention of loaded drug, the liposomes are composed predominantly of lipid components having phase transition temperatures above about 37°C.

For enhanced blood circulation time, the liposomes are coated with a hydrophilic polymer effective to extend the circulation time of the administered liposomes severalfold over that in the absence of such hydrophilic polymer.

For enhanced liposomes targeting to a solid tumor target or site of inflammation, the liposomes have sizes predominantly in the range between 0.07 and 0.12 μm.

In another general embodiment, the invention provides a liposomal composition for use in administering a compound, which includes, encapsulated within the liposomes, an ionizable carboxyl-containing compound conjugate of the compound to be delivered. The compound to be delivered possesses a derivatizable hydroxyl group, and an adduct covalently bonded to the hydroxyl group to form an ester bond, as well as a free carboxylic acid function.

Molecular adducts for use in coupling to the hydroxy-containing compound include reactive anhydrides such as succinic and glutaric anhydride, as well as di-carboxylic acids. The free acid portion of the conjugate provides efficient compound-conjugate loading by utilization of a higher inside/lower outside liposome pH gradient.

In one general embodiment, the selected pH of the internal phase of the liposomes is substantially greater than the pKa of the free carboxyl group of the carboxyl-containing compound conjugate, to maintain the compound conjugate in liposome encapsulated form.

In a related aspect, the invention includes a drug-delivery combination for use in administering to a subject, a compound having a derivatizable hydroxyl group. The combination includes a compound-conjugate composed of the compound to be administered and an adduct conjugated to the derivatizable group of such compound by an ester linkage, and liposomes, which, when suspended in an aqueous medium having a selected pH, possess an internal aqueous environment and an inside/outside pH gradient as described above. The adduct is effective to promote net uptake of the compound into the liposomes in response to the pH gradient.

In a related aspect, the invention includes a method of preparing a suspension of liposomes for use in delivering a compound having a derivatizable hydroxyl group. The method includes adding to an aqueous suspension containing (i) a bulk phase medium, and (ii) suspended in the bulk phase medium, liposomes having an internal aqueous phase, and an inside/outside pH gradient with respect to the bulk phase medium, a derivatized compound composed of the compound to be delivered, and an adduct attached to the compound's derivatizable group through an ester linkage. The compound and liposome mixture is then incubated under conditions effective to allow passage of the compound from the external medium into the liposomes, with the compound adduct being effective to produce net uptake of the compound conjugate into the liposomes in response to the pH gradient.

The incubation is preferably carried out at a temperature substantially above the phase transition temperatures of the liposome-forming lipids.

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying figures and examples.

Brief Description of the Figures

Fig. 1A shows a simplified reaction scheme for forming an ester between the 5'-OH group of a nucleoside analog and the carboxyl group of an amino acid;

Fig. 1B illustrates one a method for forming a conjugate of nucleoside analog and an amino acid;

Figs. 2A-2F show the structures of representative nucleoside-analog-amino acid conjugates useful in the invention;

Figs. 3A-3C show the structures of representative radiosensitizer-amino acid conjugates useful in the invention;

Fig. 4 illustrates the ionization events in loading a compound conjugate into pH gradient liposomes, in accordance with the invention;

Fig. 5 illustrates the ionization events in loading a compound-conjugate into liposomes, across an ammonium ion gradient; and

Fig. 6 illustrates the ionization events in loading a halodeoxyuridine/carboxyl conjugate into liposomes, across a pH gradient.

Detailed Description of the Invention

I. Definitions

The following terms, as used herein, have the meanings as indicated:

A "zwitterionic" compound, also referred to as a dipolar ion or inner salt, refers to a compound having both acidic and basic functional groups in the same molecule. A zwitterionic species possesses both a cationic and an anionic center, but exhibits no overall net charge within a selected pH range. Compounds containing both an amino group and an acid group may exist as zwitterions whenever the pKa of the protonated amino group is greater than the pKa of the acid. At pH's from about 5.5-7.5, the chief species in solution is typically the zwitterionic form of the compound itself.

"Hydroxyl-containing compound" refers to any compound which contains at least one reactive -OH group and may contain other functional moieties. A hydroxyl-containing compound may belong to any of a number of structural classes of compounds including nucleoside analogs, nitroimidazoles, anthraquinones, nitrofurans, benzoquinones, and naphthoquinones.

A hydroxyl-containing compound (RO-H) that is "substantially uncharged" at neutral pH refers to a compound which is not ionized to any appreciable extent in aqueous solution maintained within a neutral pH range, *e.g.*, 6.5-7.5. The pKa value of a hydroxy-containing compound in accordance with the invention is typically about 13 or greater. The hydroxy-containing compound may contain additional functional groups, so long as the functional groups are not ionizable within a neutral pH range, as described above.

"Compound conjugate" refers to the product formed by reacting a hydroxyl group of a compound for use in the present invention with an adduct molecule capable of forming an ester linkage. The ester bond may, in some cases, be biodegradable, *i.e.*, hydrolyzed *in vivo*. For coupling to a hydroxyl-containing compound, the adduct molecule contains at least one derivatizable carboxyl functionality or a functional group which is readily converted to a carboxyl functionality.

"Ester linkage" refers to the linkage -C(O)-OR formed by any of a number of appropriate synthetic methods including reaction between a carboxylic acid and a hydroxy-containing compound (alcohol), an acid chloride and an alcohol, or by transesterification.

"External bulk phase medium" refers to any external medium in which the liposomes are suspended.

"Molecular adduct" refers to any compound that contains at least one functional group capable of reacting with a hydroxyl function to form a covalent ester linkage. The ester bond between the compound and agent may be biodegradable, *e.g.*, can be cleaved *in vivo* by biological or physiological processes, effective to release the compound in an active form.

"Dicarboxylic acid" refers to a compound with two or more carboxylic acid groups, including protected forms thereof.

"Amino acid" refers to any compound containing both an amino group and a carboxylic acid group. Although the amino group most commonly occurs at the position adjacent to the carboxy function, the amino group may be positioned at any location within the molecule. The amino acid may also contain additional functional groups, such as amino, thio, carboxyl, carboxamide, imidazole, etc. The amino acids may be synthetic or naturally occurring, and may be used in either their racemic or optically active forms.

"Alpha carboxyl group of an amino acid" refers to the carboxyl group of an amino acid located in the position alpha or adjacent to the amino group.

"Basic amino acid" refers to an amino acid containing a second basic group, such as amino (*e.g.*, lysine), guanidino (*e.g.*, arginine), or imidazole (*e.g.*, histidine).

"Acidic amino acid" refers to an amino acid containing a second carboxyl group (*e.g.*, glutamic acid, glu) or a potential carboxyl group, such as carboxamide (*e.g.*, asparagine).

A "liposome-entrapped" or "liposome-encapsulated" compound refers to a compound which is sequestered in the internal compartment of liposomes.

A "surface coating" of hydrophilic polymer chains, such as polyethyleneglycol (PEG), on a liposome refers to the coating of any hydrophilic polymer on the surface of liposomes. The

hydrophilic polymer contains from about 20-150 monomer subunits, and more preferably, from 40-100 subunits.

"Radiosensitizer" refers to any chemical agent having the capacity to increase the lethal effects of radiation on biological cells.

"Lower inside/higher outside pH gradient" refers to a transmembrane pH gradient between the interior of liposomes (lower pH) and the external medium (higher pH) in which the liposomes are suspended. Typically, the interior-liposome pH is 1-4 pH units lower than the external-medium pH.

A "higher inside/lower outside pH gradient" refers to a transmembrane pH gradient between the interior of liposomes (higher pH) and the external medium (lower pH) in which the liposomes are suspended. Typically, the interior-liposome pH is 1-4 pH units higher than the external-medium pH.

II. Preparation of Ionizable Compound Conjugates

A. General Properties of the Molecular Adduct

In accordance with the present invention, a molecular adduct is coupled to the compound to be administered for use in improving the compound's loading profile, particularly for use in active loading against an ion or pH gradient. The adduct portion of the conjugate may be cleaved under physiological conditions to release the parent compound in an active form, such as by the action of esterases. In such instances, the active form typically corresponds to the unmodified compound.

Preferably, molecular adducts for use in the present invention are non-toxic, when administered at levels typically contained within the liposomes.

The molecular adduct may also provide additional favorable properties to the resulting conjugate, such as enhanced water solubility due to the introduction of one or more ionizable functional groups.

For adducts containing multiple functional groups, selective protection/deprotection may be required to form the desired conjugate. A variety of protected amino acids, amino acid alcohols, and activated amino acid esters may be obtained from Advanced ChemTech, Louisville, KY).

The adduct molecule is chosen to maintain the conjugate in liposome-encapsulated form in response to a selected pH gradient. The resulting derivatized compound will typically possess an overall net charge (absolute value) of one or greater within a neutral pH range.

B. AMINO ACID CONJUGATES OF HYDROXYL CONTAINING COMPOUNDS

Fig. 1A shows a general reaction scheme for preparing an amino acid conjugate of a hydroxyl-containing compound, as illustrated here by a nucleoside analog. Typical drugs in this class include nucleoside analogs, such a nitroimidazole, anthraquinones, 2-nitrofurans, benzoquinones, naphthoquinones or corticosteroids. The synthesis of various nucleoside-analog-amino acid conjugates is presented in Examples 1A-1E.

In one representative synthetic approach described in Example 1B, the hydroxyl-containing compound or active agent is esterified directly by reaction with the alpha carboxyl group of an N-protected amino acid. In coupling reactions in which the adduct is a naturally-occurring amino acid, the amino group is located adjacent the carboxy function. However, the amino group of an ionizable moiety may be located at any suitable position within the amino acid molecule.

Any of a number of amino protecting groups may be used on the adduct, such as carbobenzoxy (CBZ), tert-butoxycarbonyl (t-BOC), trityl derivatives such as trityl (Tr), dimethoxytrityl (DMTr) and the like (Greene, *et al.*). Amidine protecting groups, such as those formed by reacting an amino group with N,N-dimethylacetamide dimethylacetal, may also be used for protecting the exocyclic amino functions of various nucleosides (McBride, *et al.*, 1983; Froehler, *et al.*, 1983). Other protecting groups, such as cyclic diacyl groups or nitrophenylsulfenyl (Nps) may also provide suitable reagents for protecting amino functions. In terms of selecting a protecting group, a suitable protecting group for use in the present invention is one which is easy to introduce into the molecule, protects the amino functional group under the esterification reaction conditions, and is removable under conditions (either acidic or basic) which leave the newly formed ester linkage intact.

To favor formation of the ester, a suitable condensing agent is typically used, such as dicyclohexylcarbodiimide (DCC) or any appropriate carbodiimide reagent (Holmberg, *et al.*, 1979; Hassner, *et al.*, 1978). The condensing agent is used to activate the carboxy group of the amino acid. 4-dimethylaminopyridine (DMAP) may also be added to the reaction mixture to reduce reaction times and increase yields (Aggàrwal, *et al.*, 1990). Typically, the reactions are carried out under anhydrous conditions to disfavor the carboxylic acid; a preferred solvent is ethyl acetate. The reactions are typically carried out until complete, as determined by TLC. In carrying out direct coupling of the hydroxyl-containing compound and the amino acid by this method, the reaction times range from about 1-3 days. The N-protected compound-conjugate is then separated from the solid dicyclohexylurea by-product and purified by suitable extractive and chromatographic methods. Deblocking of the purified compound conjugate is typically carried out under acidic conditions, such as with

hydrochloric acid in dioxane. Deprotection of the amino group may also be carried out under basic conditions, depending upon the nature and sensitivity of other functional groups present in the compound-conjugate.

The preparation of exemplary amino acid conjugates of AZT and 5-bromodeoxyuridine as described above is summarized in Examples 1B and 1C, respectively. Representative amino acid adducts used to form the corresponding conjugate compounds include lysine, phenylalanine, β-alanine, glycine, and 6-aminocaproic acid. Amino acid compounds are readily available, either by synthetic or commercial (Advanced Chem Tech, Louisville, KY; Synthetech, Inc., Albany, OR; Degussa Corp., Ridgefield Park, NJ) sources.

In an alternate and somewhat preferred synthetic method, as shown in Fig. 1B and described in Example 1D, the carboxy group of the N-protected amino acid is first transformed to an active ester intermediate. In the synthetic approach illustrated, an N-protected amino acid is first reacted with 1-hydroxybenzotriazole in the presence of DCC to form the N-protected benzotriazole ester.

Any of a number of transformations may be used to activate the carboxyl group of the N-protected amino acid, for example the carboxyl group may be converted to the corresponding acyl chloride with a suitable chlorinating reagent such as phosphorus trichloride or sulfonyl chloride or the like, or converted to the corresponding anhydride.

The activated amino acid reagent, preferably the benzotriazole ester, is reacted with the hydroxyl containing compound in the presence of DMAP. A preferred solvent for carrying out the reaction is acetonitrile. Reaction times are typically less than 12 hours. After purification, the resultant N-protected compound-conjugate is deblocked under acidic conditions, preferably by mixing with trifluoroacetic acid in methylene chloride. The choice of deblocking reagent will depend upon the presence or absence of other functional groups in the compound-conjugate and their susceptibility to cleavage or reaction under the deprotecting conditions employed.

The hydroxyl-containing compound, and particularly the nucleoside analogs, may contain more than one hydroxyl moiety. In this case, and depending on the desired products, variations in the synthetic method may be employed which take into account the differences in activity of the hydroxyl groups, the proposed position for the esterification, and the like Protection of additional hydroxyl groups may be required by one of the methods commonly known in the art.

For instance, the 5-halodeoxyuridine compounds, as well as many of the nucleoside analogs, possess more than one hydroxyl group. The 5-halodeoxyuridines possess two hydroxyls, one at the 5'-position (on a primary carbon atom), and another at the 3'-position

(on a secondary carbon). For example, exclusive formation of the 3'-ester conjugate may be achieved by first protecting the 5'-OH group with an appropriate protecting group, such as trityl or one of the methoxytrityl protecting groups, followed by preparation of the desired ester at the 3' position. Conversely, exclusive formation of the 5'-ester may be promoted by first blocking the 5'-hydroxy group, followed by protection of the 3'-hydroxy function with a different protecting group. A preferred blocking group for the 5'-OH group is isobutyloxycarbonyl, while 4-methoxytrityl may be used for protection of the 3'-group (Saari, *et al.*, 1990). The di-protected alcohol is then reacted with the activating agent, such as benzotriazole or the like, which should then react preferably at the 5'-protected function of the nucleoside to form the desired product.

Figs. 2A-2F show the structures of representative nucleoside analogue-amino acid conjugates in accordance with the present invention. Fig. 2A illustrates the structure of the nucleoside analogue, minimycin, (also known as oxazinomycin) coupled to the amino acid, isoleucine. Fig. 2B illustrates a synthetic hydroxy-pyridinone, 3-deazauridine, coupled via its 5'-hydroxy function to the molecular adduct, alanine. As illustrated in Fig. 2C, the nucleoside analogue, 6-azauridine, is coupled to leucine to form an ionizable compound conjugate for efficient loading into pH gradient liposomes. Fig. 2D shows the nucleoside analogue, ARA-T, coupled to the amino acid, phenylalanine. A further example of a hydroxy-containing compound coupled to an amino acid adduct is shown in Fig. 2E, which illustrates 6-azathymidine coupled to the amino acid valine. Another exemplary compound conjugate is shown in Fig. 2F, in which the nucleoside analogue, 2'3'-didehydro-3'-deoxythymidine is coupled to the amino acid, glycine. These representative compound conjugates are efficiently loaded into pH gradient liposomes and further maintained in liposome-encapsulated form, in accordance with the present invention.

Preparation of both the 5'- and 3'- mono amino acid esters, as well as the 5',3'-diamino acid esters of 5-bromodeoxyuridine, is described in Examples 1B and 1C, respectively.

Other methods and reagents may also be employed to form the compound-conjugates of the present invention. For example, one may also use polymer-bound DMAP with DCC to promote formation of an ester linkage between a hydroxy-containing compound and a carboxylic acid. Sulfonyl chloride and sulfonyl chloride type reagents may also be used. Reagents of this type include fluorosulfonyl chloride (SO_2ClF) and triethylamine; and methylsulfonyl chloride and triethyl amine (a reagent system useful for unsaturated reactants). Phosphorus-containing reagents for use in forming esters include $PhOP(O)Cl_2$ in DMF and Bu_3PI_2 in ether and HMPT. Reagents useful in the coupling of carboxylic acids and ternary

alcohols include diimidazolylketone in DBU (1,8-diazabiclyclo[5.4.0]undec-7-ene) and $Cl(CH_3)_2NC=N(CH_3)_2^+$ in pyridine.

C. EXEMPLARY DRUGS

Among the hydroxyl containing drugs contemplated for use in the invention are:

1. Hydroxyl-containing nucleosides and nucleoside analog antiviral compounds.

A variety of hydroxyl-containing nucleoside and nucleotide analogs have activity as chemical radiosensitizers, anti-viral agents, and anti-tumor drugs. One such anti-viral nucleotide analog is ribavarin-5'-sulfamate, which has been shown to inhibit Semliki Forest virus in-vitro and in mice (Martin, J., p. 136-137, 1989). Ribovarin is a broad spectrum nucleoside analog that is active against a number of RNA and DNA viruses (Martin, J., p. 18, 19, 1989). Among the analog compounds which are useful for coupling with a molecular adduct, in accordance with the invention are:

Base-modified nucleoside analogs, such as the halogenated pyrimidines, 5-iododeoxyuridine (IUdR), 5-bromodeoxyuridine (BUdR), 5-chlorodeoxyuridine (CUdR), and 5-fluorodeoxyuridine (FUdR), trifluorothymidine, dideoxyinosine (DDI), minimycin, 3-deazauridine, 6-azathymidine, and ribavirin. Trifluorothymidine and IUdR are active mainly against herpes viruses (Nicholson, 1984a, 1984b). Ribavirin is active against several RNA and DNA viruses (Sidwell). The halogenated 5-deoxyuridine compounds, IUdR, BUdR, CUdR, and FUdR, are modifiers of radiosensitivity but are not specific to hypoxic cells (Shenoy, *et al.*, 1992). These compounds are incorporated into the DNA chain in place of thymidine, thereby rendering the DNA more susceptible to radiation treatment.

Other nucleoside analogs include sugar-modified nucleoside analogs, such as N-acyl derivatives of 5'-amino-2',5'-dideoxy 5'-iodouridine, 3'-azido-2',3'-dideoxythymidine (AZT), acyclovir, ganciclovir, dideoxycytidine (DDC), dideoxyinosine (DDI, both base and sugar modified), 2',3'-didehydro-3'-deoxythymidine (D4T), sulfonamide derivatives of 5'-amino-5'-deoxythymidine, 2'-deoxy-5-ethyluridine, and N-acyl derivatives, AraA, ARA-T, 5'-sulfate and 5'-sulfamate nucleoside analogs, such as nucleocidin, adenosine 5' sulfamate, and ribavarin-5-sulfamate, which may act primarily at the level of protein synthesis inhibition (Martin, J.D., 1989). ARA-T has been reported to inhibit the replication of HSV-1 and HSV-2 (Walker); d4T has been shown to inhibit the replication of HIV. AZT is active against HIV (Fischl), as are other dideoxynucleoside analogs, such as DDI. Acyclovir is active against herpes simplex 1 and 2 and varicella zoster (Fishl, *et al.*, 1987); ganciclovir,

an analog of acyclovir, is active against HSV-1, HSV-2, varicella zoster as well cytomegalovirus, CMV (Smith, *et al.*, 1982).

Nucleoside phosphate/phosphonate analogs include acyclonucleoside phosphonates and phosphates such as those of acyclovir and ganciclovir. These compounds can act as virus-selective substrates for viral thymidine kinases in the synthesis of nucleoside triphosphate analogs intracellularly (Galbraith, *et al.*, 1969). Subsequently, the nucleoside triphosphate analogs can act as selective substrates for viral DNA polymerase, thereby functioning as chain terminators due to their lacking the bifunctionality necessary for chain extension (Allen, *et al.*, 1983). These compounds have demonstrated anti-viral activity against herpes viruses (Collins, *et al.*, 1979), including HSV-1, HSV-2, varicella zoster (VZV), and cytomegalovirus (CMV) (Smith, *et al.*, 1982).

Also included in this class are phosphonomethyl ethers of nucleosides, and their acyclic analogs, such as N-(3-hydroxy-2-phosphonylmethoxypropyl)- (HPMP-) and N-(2-phosphonylmethoxyethyl-)(PME-) derivatives of heterocyclic bases. These compounds act specifically against herpes viruses, adenoviruses, cytomegalovirus (DeClercq, 1988), poxviruses, vaccinia viruses, and retroviruses.

2. Radiosensitizers.

Radiosensitizers are chemical agents that have the capacity to increase the lethal effects of radiation when administered in conjunction with radiation, typically for use in cancer therapy. Ideally, they should increase radiation sensitivity without being toxic.

Although shown to be effective radiosensitizers, the potential usefulness of the 5'-halodeoxyuridines is limited by a number of factors (Shenoy, *et al.*, 1992). In order to provide effective therapy, the drugs must be present in the bloodstream for a period long enough to allow the cells to pass through at least one DNA synthesizing cycle. Secondly, the rapid hepatic degradation and dehalogenation must be overcome. The liposome composition of the invention, and particularly the liposome formulation employing hydrophilic polymer derivatized lipids, provides an enhanced mode of delivery of this class of radiosensitizers by providing enhanced circulation times and protecting the compound from dehalogenation by encapsulation within the liposome bilayer.

In addition to the halogenated 5'-deoxyuridines, several other types of compounds are known to possess radiosensitizing activity. Many of these compounds possess functional groups which can act as electron acceptors, such as carbonyl, aldehyde, nitro, or cyano groups which are attached to an aromatic or heterocyclic ring. These electron-affinic compounds are specific for hypoxic tumor cells (Adams, 1992).

Similarly, bioreductive drugs, compounds that are chemically reduced intracellularly to form active cytotoxic agents, are suitable for use with the present invention. Often, compounds may function as both radiosensitizers and bioreductive agents. Bioreductive compounds may be useful as agents in selective chemotherapy targeted against solid tumors (Adams, 1992). This is due to the fact that bioreduction is often favored under hypoxic conditions and hypoxic cells have been shown to exist in most murine tumors and several human tumors (Shenoy, *et al.*, 1992).

Classes of compounds which are classified as either radiosensitizers or bioreductive compounds include nitro-containing heterocycles such as the nitroimidazoles (*e.g.*, metronidazole, misonidazole, etanidazole, nimorazole); nitrofurans (*e.g.*, nifurpipone); fused ring quinones such as benzoquinones, anthraquinones, naphthoquinones and the like (*e.g.* E09); and organic N-oxides. Representative radiosensitizer compound-amino acid conjugates useful in the present invention are shown in Figs. 3A-3C. The exemplary radiosensitizer compounds illustrated are 5-iododeoxyuridine, misonidazole, and E09 (Adams), respectively.

3. Anti-Inflammatory Compounds.

A third general class of compounds include anti-inflammatory compounds, including steroidal anti-inflammatory drugs. Steroidal compounds for use in the invention include the hydroxyl-containing corticosteroidal compounds, prednisone, methylprednisolone, paramethazone, 11-fludrocortisol, triamcinolone, betamethasone and dexamethasone.

Such anti-inflammatory compounds, when coupled through a reactive group to an adduct molecule effective to maintain the conjugate in liposome entrapped form, are suitable for delivery by the liposome composition of the present invention, particularly by IV administration. The compound is useful, preferably, for treating inflammation and particularly for inflamed dermal tissue.

Compounds of the types described above are substantially uncharged at a neutral pH and contain a hydroxyl functionality that can be derivatized, as above, to produce compound conjugates effective for loading into pH gradient liposomes of the type now to be described.

III. pH Gradient Liposomes

This section describes the preparation of liposomes having an inside/outside pH gradient for active loading of conjugate compounds of the type described above.

A. LIPID COMPONENTS

The liposomes of the invention are composed of vesicle-forming lipids, generally including amphipathic lipids having both hydrophobic tail groups and polar head groups. A characteristic of a vesicle-forming lipid is its ability to either (a) form spontaneously into bilayer vesicles in water, as exemplified by the phospholipids, or (b) be stably incorporated into lipid bilayers, by having the hydrophobic portion in contact with the interior, hydrophobic region of the bilayer membrane, and the polar head group oriented toward the exterior, polar surface of the membrane.

The vesicle-forming lipids of this type are preferably those having two hydrocarbon tails or chains, typically acyl groups, and a polar head group. Included in this class are the phospholipids, such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylglycerol (PG), and phosphatidylinositol (PI), where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. Preferred phospholipids include PE and PC. An exemplary PC is hydrogenated soy phosphatidylcholine (HSPC). Single chain lipids, such as sphingomyelin (SM) may also be used.

The above-described lipids and phospholipids whose acyl chains have a variety of degrees of saturation can be obtained commercially, or prepared according to published methods. Other lipids that can be included in the invention are glycolipids. The term "glycolipid" as used herein encompasses lipids having two hydrocarbon chains, one of which is the hydrocarbon chain of sphingosine, and one or more sugar residues.

Lipids for use in the present invention may be relatively "fluid" lipids, meaning that the lipid phase has a relatively low lipid melting temperature, *e.g.*, at or below room temperature, or alternately, relatively "rigid" lipids, meaning that the lipid has a relatively high melting point, *e.g.*, at temperatures up to 50°C. As a general rule, the more rigid, *i.e.*, saturated lipids, contribute to greater membrane rigidity in the lipid bilayer structure, and thus to more stable drug retention after active drug loading. Preferred lipids of this type are those having phase transition temperatures above about 37°C.

The liposomes may additionally include lipids that can stabilize a vesicle or liposome composed predominantly of phospholipids. The most frequently employed lipid from this group is cholesterol at levels between 25 to 40 mole percent. When cholesterol is present at between 0 to 20 mole percent in a bilayer, undesirable separate domains containing cholesterol and phospholipids and pure phospholipid may exist (Mabrey, *et al.*, 1978). These bilayers show an increased permeability to water (Tsong, 1975). At mole percentages above 50%, cholesterol may contribute to destabilizing the bilayer.

The liposomes may also include a coating of a hydrophilic polymer chain. Liposomes thus prepared may include one or more vesicle-forming lipids derivatized with a hydrophilic polymer chain. The vesicle-forming lipids which can be used are any of those described above for the first vesicle-forming lipid component. Vesicle-forming lipids with diacyl chains, such as phospholipids, are preferred. One preferred phospholipid is phosphatidylethanolamine (PE), which contains a reactive amino group convenient for coupling to the activated polymers. One exemplary PE is distearyl PE (DSPE).

A preferred hydrophilic polymer for use in coupling to a vesicle forming lipid is polyethyleneglycol (PEG), preferably as a PEG chain having a molecular weight between 1,000-10,000 daltons, more preferably between 2,000 and 5,000 daltons. Examples 2 and 3 present the synthesis of one preferred liposome-type for use in the present invention, namely, long circulating liposomes composed of PEG-PE, HSPC and cholesterol. The details of the synthesis are described therein.

Other hydrophilic polymers which may be suitable include polylactic acid, polyglycolic acid, polyvinylpyrrolidone, polymethyloxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide, polydimethylacrylamide, and derivatized celluloses, such as hydroxymethylcellulose or hydroxyethylcellulose. Lipid-polymer conjugates containing these polymers attached to a suitable lipid, such as PE, are described in co-owned U.S. Patent No. 5,395,619, (issued on 7 March 1995).

An exemplary method of preparing a PE-PEG compound is described in Examples 2A and 2B. Briefly, a capped polyalkylether, polyethylene glycol methyl ether, is first activated with a carbonyl diimidazole reagent to form the activated carbamate. Reaction with an amino-containing lipid such as PE leads to coupling of the PEG to the lipid through formation of amide, carbamate or urethane bonds.

In formed liposomes, the hydrophilic polymer chains provide a surface coating of hydrophilic chains sufficient to extend the blood circulation time of the liposomes in the absence of such a coating. The extent of enhancement of blood circulation time is severalfold over that achieved in the absence of the polymer coating, as described in co-owned US patent No. 5,013,556.

Further, the liposomes may be prepared to contain surface groups, such as antibodies or antibody fragments, small effector molecules, and the like, for achieving desired target-binding properties.

B. PREPARING pH GRADIENT LIPOSOMES

Liposomes with the desired pH gradient may be prepared by a variety of techniques. In a typical procedure, a mixture of liposome-forming lipids is dissolved in a suitable organic solvent and evaporated in a vessel to form a thin film. The film is then covered with an aqueous medium containing the solute species that will form the aqueous phase in the liposome interior spaces, in the final liposome preparation, as discussed below. The lipid film hydrates to form multi-lamellar vesicles (MLVs), typically with heterogeneous sizes between about 0.1 to 10 microns.

Liposomes used in the invention typically contain between 30-75 percent phospholipids, preferably phosphatidylcholine (PC), 25-40 percent cholesterol, and 1-20 percent polymer-derivatized lipid, expressed on a molar percent basis. One exemplary "rigid" liposome formulation is formed of polyethylene glycol conjugated phosphatidylethanolamine, PEG-PE, hydrogenated soy phosphatidylcholine (HSPC) and cholesterol in a weight ratio of 1:3:1. Two exemplary fluid liposome compositions contain the components PEG-PE, egg phosphatidylcholine (EPC), and cholesterol, in corresponding molar ratios of 6:56:38 and 6:62:32.

After liposome formation, the vesicles may be sized to achieve a size distribution of liposomes within a selected range, according to known methods. The liposomes are preferably uniformly sized to a selected size range between 0.04 to 0.25 μm. Small unilamellar vesicles (SUVs), typically in the 0.04 to 0.08 μm range, can be prepared by extensive sonication or homogenization (Martin, 1990) of the liposomes. Homogeneous size liposomes having sizes in a selected range between about 0.08 to 0.4 microns can be produced, *e.g.*, by extrusion through polycarbonate membranes or other defined pore size membranes having selected uniform pore sizes ranging from 0.03 to 0.5 microns, typically, 0.05, 0.08, 0.1, or 0.2 microns. The pore size of the membrane corresponds roughly to the largest size of liposomes produced by extrusion through that membrane, particularly where the preparation is extruded two or more times through the same membrane.

The sizing is preferably carried out in the original lipid-hydrating buffer, so that the liposome interior spaces retain this medium throughout the initial liposome processing steps.

Liposomes having a lower inside/higher outside pH gradient are typically prepared as described below. After sizing, the liposome suspension is treated to adjust the pH of the external medium to a selected pH above that of the internal pH, or to replace the external bulk-phase medium with a medium which will create the desired lower inside/higher outside pH gradient in the final suspension. Exchange of the external bulk-phase medium in a liposome suspension is carried out conventionally, *e.g.*, by dialysis against the desired final

medium, molecular sieve chromatography in the desired medium, e.g., using Sephadex G-50, or high-speed centrifugation and resuspension of pelleted liposomes in the desired final medium.

The external medium which is selected will depend on the mechanism of pH gradient formation and the external pH desired, as will now be considered.

In the simplest approach for generating a pH gradient, the hydrated sized liposomes have a selected internal-medium pH. The suspension of the liposomes is titrated until a desired final pH is reached, or treated as above to exchange the external phase buffer with one having the desired external pH. For example, the original medium may have a pH of 5.5, in a selected buffer, *e.g.*, glutamate or phosphate buffer, and the final external medium may have a pH of 8.5 in the same or different buffer. The internal and external media are preferably selected to contain about the same osmolarity, e.g., by suitable adjustment of the concentration of buffer, salt, or low molecular weight solute, such as sucrose.

In another general approach, the gradient is produced by including in the liposomes, a selected ionophore. To illustrate, liposomes prepared to contain valinomycin in the liposome bilayer are prepared in a potassium buffer, sized, then exchanged with a sodium buffer, creating a potassium inside/sodium outside gradient. Movement of potassium ions in an inside-to-outside direction in turn generates a lower inside/higher outside pH gradient, presumably due to movement of protons into the liposomes in response to the net electronegative charge across the liposome membranes (Deamer).

In a preferred embodiment, the proton gradient used for drug loading and retention is produced by creating an ammonium ion gradient across the liposome membrane, as described, for example, in U.S. Patent No. 5,192,549. Here the liposomes are prepared in an aqueous buffer containing an ammonium salt, typically 0.1 to 0.3 M ammonium salt, such as ammonium sulfate, at a suitable pH, *e.g.*, 5.5 to 7.5. After liposome formation and sizing, the external medium is exchanged for one lacking ammonium ions, e.g., the same buffer but one in which ammonium sulfate is replaced by NaCl or a sugar that gives the same osmolarity inside and outside.

As shown in Fig. 5, ammonium ions inside the liposomes are in equilibrium with ammonia and protons. This equilibrium is continuously shifted toward production of protons by the escape of ammonia from the liposome interiors.

The ammonium ion gradient provides a number of advantages in active drug loading over the other two approaches given above. Among these are:

1. The system is able to generate protons in response to drug loading, such that the ability to load drugs is not limited by the initial concentration of protons or initial pH

gradient. That is, as drug molecules in non-protonated form are taken up and protonated within the liposomes, the loss of protons within the liposomes shifts the ammonium/ammonia equilibrium toward ammonia and proton production, maintaining the concentration of protons at a relatively constant level independent of amount of drug loaded. The only requirement is that the initial ammonium ion concentration within the liposomes is in substantial molar excess of the final loaded drug concentration.

2. The system is able to act like a battery during liposome storage, replacing protons that may be lost by diffusion out of the membranes with newly produced protons, as the ammonium/ammonia equilibrium is shifted toward ammonia production. This allows for stable drug retention in liposomes for periods of up to several months in a liquid-suspension form.

3. The counterion of the ammonium salt, *e.g.*, sulfate counterion, may further enhance drug loading, by its ability to precipitate or form insoluble complexes with the drug being loaded.

IV. Drug Loading into Liposomes

This section describes the compound loading method of the invention, and the composition formed by the method.

A. Compound Loading Method

The method includes adding to an aqueous suspension of pH gradient liposomes of the type described above, a derivatized compound composed of the compound to be delivered, and an adduct attached to the compound's derivatizable group through an ester linkage.

In the loading method, the components are incubated under conditions effective to allow passage of the compound from the external medium into the liposomes, where the presence of said adduct is effective to produce net uptake of the compound conjugate into the liposomes in response to said pH gradient. That is, the compound in the absence of the adduct is not actively loaded into such liposomes or is loaded relatively poorly.

Fig. 4 illustrates the manner of drug loading of a derivatized drug conjugate into liposomes containing a pH gradient. The derivatized compound here is shown with an amine adduct that thus provides an ionizable group capable of forming a positive charge. As shown, the drug exists in equilibrium with its protonated ammonio form in the external liposomal environment.

In response to the excess of protons accumulated in the liposome interior, neutral-charge drug conjugate diffusing across the liposomal membrane is shifted toward its protonated, charged, non-diffusible form, and thus accumulates in the liposomes.

Fig. 5 illustrates loading of a derivatized drug, exemplified by a halo-deoxyuridine-amine conjugate into liposomes against an ammonium ion gradient. As noted above, the gradient is effective to generate excess internal protons through a mechanism involving dissociation of ammonium and escape of ammonia. When the derivatized compound in uncharged form enters the liposomes, its equilibrium is shifted toward a protonated form, due to the higher concentration of internal protons, and in this charged form, leading to net accumulation of compound in the liposomes.

If, as illustrated in the figure, the protonated form of the drug can form a complex or precipitate with the ammonium salt counterion (Cl^-), the compound is further driven toward an entrapped state, increasing the concentration of compound that can be entrapped, and the stability of drug entrapment. The solubility of a derivatized compound in various ammonium-salt counterions can be determined by standard methods, for purposes of selecting a counterion which will lead to complex formation and enhanced drug entrapment.

Incubation conditions suitable for drug loading are those which (i) allow diffusion of the derivatized compound, with such in an uncharged form, into the liposomes, and (ii) preferably lead to high drug loading concentration, e.g., 50-200 mM drug encapsulated. The loading is preferably carried out at a temperature above the phase transition temperature of the liposome lipids. Thus, for liposomes formed predominantly of saturated phospholipids, the loading temperature may be as high as 60°C or more. The loading period is typically between 15-120 minutes, depending on permeability of the derivatized drug to the liposomes, temperature, and the relative concentrations of liposome lipid and drug.

With proper selection of liposome concentration, external concentration of added compound, and the pH gradient, essentially all of the derivatized compound may be loaded into the liposomes. For example, with a pH gradient of 3 units (or the potential of such a gradient employing an ammonium ion gradient), the final internal:external concentration of drug will be about 1000:1. Knowing the calculated internal liposome volume, and the maximum concentration of loaded drug, one can then select an amount of drug in the external medium which leads to substantially complete loading into the liposomes.

Alternatively, if drug loading is not effective to substantially deplete the external medium of free drug, the liposome suspension may be treated, following drug loading, to remove non-encapsulated drug. Free drug can be removed, for example, by molecular sieve chromatography, dialysis, or centrifugation.

Example 4A describes a method for preparing rigid and fluidic PEG-coated liposomes having an ammonium sulfate gradient of about 250 mM $[NH_4]_2SO_4$ inside, and near 0 mM ammonium salt outside. The liposomes were loaded in the presence of an equal concentration of either AZT-amino acid conjugate or 5'-bromodeoxyuridine-amino acid conjugate in 10% sucrose buffer, at final drug concentrations of 2.5 mg/ml. After loading and removal of free compound-conjugate, the liposomes were analyzed to determine *i)* the percent of encapsulated nucleoside analog-amino acid conjugate, and *ii)* percent of nucleoside analog-amino acid conjugate recovered. The loading efficiencies are reported in Tables I and II in Example 4.

In a related aspect, the invention includes the combination of the above-described pH gradient liposomes and derivatized compound for use in the method. The two components are typically supplied and stored separately, for combining at the site of intended use, *e.g.*, a clinical site, to yield a suspension of compound-loaded liposomes.

The combination is advantageous particularly where the drug-loaded liposomes have a relatively high rate of drug leakage, after loading, precluding extended storage time in liquid suspension, or where it is impractical to store the drug-loaded liposomes in dried, *e.g.*, lyophilized form.

B. Compound-Loaded Liposomes

Where the compound-loaded liposome composition prepared as above is stable on storage in liquid suspension form over an extended period, e.g., several months, it may be supplied and stored in this preloaded form.

Factors which contribute to storage stability in suspension are: (i) compound stability, both in terms of the ester bond used for compound derivatization and stability of the compound itself against oxidative or other degradative processes in solution; (ii) liposome stability in suspension, typically related to the degree of lipid saturation, and (iii) slow compound leakage from the liposomes.

Drug stability in solution can be determined from stability studies, e.g., under accelerated conditions of elevated temperature, according to known procedures. If necessary, drug-protective agents, such as α-tocopherol, may be added to enhance compound stability on storage.

Liposome stability in liquid suspension can be enhanced by the use of saturated, high-phase transition lipid components, and optionally by inclusion of such protective agents as α-tocopherol or an iron-chelating agent, such as desferioxamine.

Drug leakage from liposomes can be minimized by use of high-phase transition lipids, as discussed above, by maintaining excess protons in the encapsulated space and, if possible, by

employing an internal salt counterion that is effective to remove encapsulated compound out of the solute phase.

Alternatively, the composition containing a derivatized compound loaded within its interior spaces may be stored in lyophilized form. The composition here preferably includes a cryoprotectant, such as a mono- or disaccharide, added to the external medium.

V. Reverse-pH Method and Composition

According to another embodiment of the invention, a hydroxyl-containing compound to be delivered by liposomes is modified to contain a carboxy group linked to the compound through an ester linkage. This allows loading of the transformed, ionizable compound against a higher inside/lower outside liposome pH gradient, or a reverse pH gradient.

A. Carboxyl Conjugates of Hydroxyl-Containing Compounds

The hydroxyl-containing drug is modified by linking the drug's hydroxyl-group to a di-carboxylic acid or a similarly suitable difunctional adduct. A suitable adduct for use in this aspect of the invention is one: *i)* that possesses at least one functional group capable of reacting with a drug hydroxyl group to form an ester linkage, and *ii)* has at least one carboxylic acid group, or results in a carboxyl group upon coupling to the drug molecule. Such molecules include dicarboxylic acids and reactive cyclic anhydrides, such as succinic, glutaric and phthalic anhydride.

The resulting conjugate, possessing an ionizable carboxy group, is maintained in liposome-encapsulated form by selection of an appropriate pH gradient. Preferably, the pH of the internal phase of the liposomes is substantially greater than the pKa of the conjugate carboxylic acid group, and preferably by at least 1 to 2 pH units.

One method for modifying a hydroxy-containing compound to a carboxyl-containing molecule is to couple the compound with an ionizable moiety such as a di-carboxylic acid (or the corresponding cyclic anhydrides, such as glutaric or succinic anhydride) to form the corresponding mixed acid-ester. One of the advantages of this approach, in addition to being able to utilize pH gradient loading, is the potentially biodegradable nature of the ester linkage. The resulting ester bond may be sensitive to acidic conditions, such as the endosomal compartment of cells to which such liposomes are targeted. Upon hydrolysis in an acidic environment, the conventional compound may then be released in its active form to the desired target site.

In preparing the carboxy-containing conjugate by reaction with a dicarboxylic acid, typically, one of the acid groups of the diacid adduct is protected with a suitable protecting

group to prevent ester formation at both reactive -COOH moieties of the adduct. Choice of a suitable protecting group will be guided by the consideration that the protecting group must be one that is removable under conditions that leave the newly formed ester-bond between the hydroxy-compound and the molecular adduct intact. Subsequent to coupling the adduct to the hydroxy-containing drug, the protecting group is cleaved from the adduct portion of the conjugate to generate a free carboxylic acid group, which may then be utilized to promote active ion-gradient loading as described above.

Alternatively, a cyclic acid anhydride reagent may be used for coupling to the drug, to form the mixed acid-ester conjugate. Synthesis of the corresponding 5'-,3'-difunctionalized conjugates formed by reacting iododeoxyuridine with either glutaric or succinic anhydride is described in Examples 5A and 5B, respectively. The resulting conjugates possess carboxyl-containing side chains at both the 5'- and 3'- positions of the 2'-deoxyribose ring. The monofunctionalized 5'- or 3'- conjugates may also be formed by the use of suitable protecting groups, as described above. The carboxylic acid groups of the resulting conjugates are separated from the ester carbonyl carbons by either two or three intervening methylenes, depending upon the choice of cyclic anhydride reagent.

As described in Examples 5A and 5B, formation of the di-glutaryl and di-succinyl adducts of iododeoxyuridine may be afforded by reacting iododeoxyuridine with either glutaric or succinic anhydride in the presence of DMAP.

Alternatively, compounds containing a primary hydroxyl functionality may be oxidized to the corresponding carboxylic acid; secondary and tertiary hydroxyl groups are unaffected. Such compounds may be transformed to possess a carboxy group either for coupling via an ester linkage to a suitable hydroxy-containing adduct or to introduce an ionizable function to the compound to allow liposomal loading against a pH gradient. Oxidants for this purpose include permanganate salts such as sodium permanganate, potassium permanganate and copper permanganate.

This approach may also be utilized to transform aldehyde compounds to the corresponding acids. Reagents for this purpose include sodium permanganate, $Ca(OCl)_2$, $(bipy)H_2CrOCl_5$, and the like. For compounds which are irreversibly derivatized, such as by reaction with oxidants, the safety and efficacy of the resulting modified compound should be verified prior to liposome loading for use in administering to a subject.

B. Reverse pH Gradient Liposomes

Liposomes having a reverse pH gradient are prepared substantially as above, but under conditions that lead to a reverse gradient. In one general method, the liposomes are prepared

by hydration in the higher pH medium, *e.g.*, pH 6-9, then treated to adjust the external pH to the selected lower pH. This may be accomplished by addition of an acid to the desired pH. Alternatively, the external medium of the liposomes suspension is exchanged with a lower pH medium.

Preferably, the pH of the internal phase of the liposomes is substantially greater than the pKa of the free carboxylic acid group, and preferably by at least 1-2 units above this pK_a value. The external medium is likewise below this pK_a.

In another embodiment, the liposomes are prepared in the presence of a bicarbonate salt, *e.g.*, 200 mM sodium bicarbonate at a selected pH, *e.g.*, 5-9. After liposome formation and sizing, the external medium is exchanged for buffer lacking bicarbonate ions, *e.g.*, the same buffer, but one in which sodium bicarbonate sulfate is replaced by NaCl or a sugar that gives the same osmolarity inside and outside.

A reverse gradient may be established by a number of different mechanisms. For instance, bicarbonate ions inside the liposomes are in equilibrium (to a small degree) with CO_2 (arising from the corresponding conjugate acid, carbonic acid) and hydroxyl ions. The equilibrium may be shifted toward production of hydroxyl ions by the escape of CO_2 from the liposome interiors, thus generating a higher internal pH. Carbonic acid, due to its small size, may also readily diffuse out of the liposome interior, also shifting the equilibrium towards hydroxyl production.

Depending upon the pH of the liposome interior, bicarbonate, in the presence of excess protons (or a source of protons, such as from the compound conjugate), is in equilibrium with carbonic acid. As described above, loss of carbonic acid, either by conversion to carbon dioxide gas or diffusion, shifts the equilibrium towards consumption of bicarbonate ions and provides a driving force for loading high concentrations of drug conjugate. Additionally, the accumulation of sodium counterions in the liposome interior may result in an outflow of protons, resulting in a liposome interior having a lower concentration of protons than in the external bulk phase.

The bicarbonate ion gradient provides advantages in active drug loading, in reverse pH gradient liposomes, similar to the advantages provided by an ammonium ion gradient. In particular:

1. The system is able to generate hydroxyl ions in response to drug loading, such that the ability to load drugs is not limited by the initial concentration of hydroxyl ions or the initial pH gradient.

2. The system is able to function like a battery during liposome storage, either by replacing hydroxyl ions that may be lost by diffusion out of the membranes with newly produced ions, or by producing an outflow of protons, as the bicarbonate/CO_2 equilibrium is shifted toward CO_2 production.

C. COMPOUND LOADING

The carboxyl-containing drug, initially present only in the external liposomal environment and primarily in its acid form (uncharged), diffuses readily into the liposomes, where the higher internal pH shifts the ionization state of the compound toward its charged, deprotonated form. The loading events are illustrated in Fig. 6 for loading of a representative halodeoxyuridine carboxy conjugate. Loading conditions are substantially like those described above in Section IV.

From the foregoing, it can be appreciated how various objects and features of the invention are met. The invention allows a variety of important classes of therapeutic drugs to be loaded into liposomes at substantially higher loading efficiencies and internal drug concentrations than has been possible heretofore.

The invention allows for remote compound loading at the site, such that the compound to be loaded, and optionally, pH gradient liposomes, can be supplied and stored in lyophilized form and loaded to high compound concentration directly before use.

Finally, compounds administered in accordance with the invention are in an esterified form which may be designed, by suitable adduct selection, to enhance the pharmacokinetic properties of the drug and/or reduce side effects.

The following examples illustrate, but are in no way are intended to limit the scope of, the present invention.

Materials and Methods

The N-protected t-BOC amino acids lysine, phenylalanine, glycine, and β-alanine, as well as t-BOC aminocaproic acid were purchased from Sigma (St. Louis, MO). Iododeoxyruridine was purchased from Sigma. 1,3-dicyclohexylcarbodiimide was purchased from Aldrich (Milwaukee, WI). Glutaryl anhydride and succinic anhydride were purchased from Lancaster (Windham, NH). DMAP was purchased from Nepera, Inc. (Harriman, NY).

Polyethylene glycol methyl ether 1900 was purchased from Aldrich Chemical Company. Cholesterol was obtained from Sigma. Egg phosphatidylcholine (EPC), partially hydrogenated soy PC (HSPC) having the compositions IV40, IV30, IV20, IV10, and IV1, phosphatidylglycerol (PG), phosphatidylethanolamine (PE), dipalmitoyl-phosphatidyl glycerol

(DPPG), dipalmitoyl PC (DPPC), dioleyl PC (DOPC), and distearoyl PC (DSPC) were obtained from Avanti Polar Lipids (Birmingham, Ala) or Austin Chemical Company (Chicago, Ill).

^{1}H NMR spectra of the nucleoside-amino acid esters were obtained using a Nicolet spectrometer operating at 360 mHz. Samples were dissolved in deuterated chloroform or deuterated methanol, tetramethylsilane (TMS) was used as an internal standard.

Liposome particle size distribution measurements were obtained by dynamic light scattering (DLS) using a Coulter N4MD instrument.

Example 1

Preparation of Nucleoside Analog-AminoAcid Conjugates

A. General Procedure

For the nucleoside-amino acid coupling reactions, control experiments were first carried out to examine the possibility of side reactions occurring at the heterocyclic moieties of AZT and bromodeoxyuridine and to investigate the possibility of side reactions involving the 5-bromo substituent of BrdU.

One set of reactions was carried out as in Example 1D except for the ratios of reactants used. The protected amino acid, t-BOC-phenylalanine, was used as the sole amino acid reagent. The following ratios of amino acid-to-BrdU were used: 0.2, 0.4, 0.6, and 0.8. The reaction products were consistent with those observed in Examples 1B, and 1D.

In order to accurately identify the products formed in Examples 1A-D, and to examine the stability of the 3'-azido function of AZT and the 5-bromo substituent in BrdU, similar coupling reactions were performed with the nucleosides uracil, thymine, deoxyuridine, and thymidine. t-BOC phenylalanine was used as the amino acid reagent. The resultant products supported the stability of both the sugar and heterocyclic rings of AZT and BrdU under the reaction conditions employed.

B. Preparation of 5'-amino acid esters of 3'-azido-2',3'-dideoxythymidine (AZT)

The 5'-OH function of AZT was esterified by reaction with various N-protected amino acids: lysine, phenylalanine, B-alanine, glycine, as well as with 6-aminocaproic acid. Protection of the free amino functions was afforded by using the corresponding t-BOC-amino acids (t-BOC = tert-butoxycarbonyl) for carrying out the coupling reactions. The reactions were carried out under anhydrous conditions using dicyclohexylcarbodiimide (DCC) as the condensing agent in the presence of 4-dimethylaminopyridine (DMAP) as a catalyst.

1.2 equivalents of DCC were added to a stirred solution containing 1.0 equivalents of AZT, 1.1 equivalents of the t-BOC amino acid, and 1.5 equivalents of DMAP in ethyl acetate. The reaction mixture was stirred for about 3 days at room temperature.

The resultant mixture was then filtered to remove the dicyclohexylurea side product and the filtrate was washed sequentially to remove unwanted impurities. Residual amino acid was extracted from the reaction solution with a 0.2 M carbonate/bicarbonate solution, followed by washing with a 0.2 M sodium chloride solution. Unreacted DMAP was extracted by washing with an aqueous solution of citric acid. The product was purified by column chromatography over silica gel using an eluent gradient of 1:1, 2:1, 4:1, 6:1, and 8.5:1 ethyl acetate-hexane. The purified ester conjugate was deblocked by reaction with 4 M HCl in dioxane. The products were characterized primarily by thin layer chromatography (visualization by UV, ninhydrin), and proton NMR spectroscopy. Yields of the AZT-amino acid esters formed by coupling AZT with lysine, phenylalanine, glycine, alanine, and 6-aminocaproic acid, respectively: 3'-azido-3'-deoxy-5'-O-(2,6-diaminohexanoyl)thymidine, 3'-azido-3'-deoxy-5'-O-(2-amino-3-phenylpropanoyl)thymidine, 3'-azido-3'-deoxy-5'-O-(aminoethanoyl)thymidine, 3'-azido-3'-deoxy-5'-O-(2-aminopropanoyl)thymidine, 3'-azido-3'-deoxy-5'-O-(6-aminohexanoyl)thymidine ranged from 50-85%.

C. Preparation of the 5'-Amino Acid Esters, 3'-Amino Acid Esters, and 5',3'-Diamino Acid Esters of 5-Bromodeoxyuridine

Esterification of 5-bromodeoxyuridine with various amino acids was carried out as described in Example 1A. Since 5-bromodeoxyuridine contains two reactive hydroxyl groups and neither group was protected prior to carrying out the coupling procedure, mixtures of both the 5'-, and 3'-mono amino acid esters and the corresponding 5',3'-diamino acid esters were obtained. Yields ranged from 50-85%.

D. Modified Preparation of 5'-Amino Acid Esters of 3'-Azido-2',3'-Dideoxythymidine (AZT)

In an alternative approach, the 5'-amino acid esters of AZT were prepared by reacting AZT with an activated amino acid ester to improve reaction times and yields. The N-protected t-BOC amino acids used were the same as those in Example 1A.

In a typical procedure, 1 mM of t-BOC amino acid and a slight excess (1.2 mM) of 1-hydroxybenztriazole (HOBt) were dissolved in approximately 20 mL of ethyl acetate. Upon dissolution of the reagents, 1.2 mM of DCC was added to the solution and the resulting mixture allowed to stir for a minimum of four hours at room temperature. Formation of the

N-protected benzotriazole ester was indicated by formation of a solid white precipitate by-product, 1,3-dicyclohexylurea.

Upon completion of the reaction, the dicyclohexylurea by-product was removed by filtration and the resultant reaction mixture was extracted with a 1:1 solution of 1 M carbonate/bicarbonate at pH 10 to remove any remaining impurities, as determined by TLC. The organic phase was dried over magnesium sulfate. The purified ester was recovered as a white crystalline solid by removal of ethyl acetate solvent by evaporation under reduced pressure. Reaction of t-BOC glycine with HOBT failed to form the desired activated ester.

The isolated benzotriazole ester was then dissolved in acetonitrile and reacted with AZT in the presence of DMAP by stirring for about 12 hours at room temperature. Upon completion, the reaction mixture was evaporated to dryness and the crude product was dissolved in a minimal amount of methylene chloride. The organic phase was extracted with a 1 M solution of carbonate/bicarbonate, followed by extraction with a 10% aqueous solution of citric acid to remove impurities. The methylene chloride layer was dried over magnesium sulfate and the solvent removed by evaporation. The N-protected conjugate product was determined to be essentially pure by TLC.

To deblock the N-protected conjugate, the N-protected amino acid-AZT ester was dissolved in a minimum volume of a 1:1 trifluoroacetic acid (TFA):methylene chloride solution and stirred at room temperature for at least 15 minutes. The reaction was continued until the blocked amino acid ester was no longer visible by TLC. Upon completion of the reaction, the reaction mixture was evaporated to dryness. A minimum volume of benzene was added and residual water removed by evaporation of the benzene-water azeotrope.

E. <u>Modified Preparation of the 5'-Amino Acid Esters, 3'-Amino Acid Esters, and 5',3'-Diamino Acid Esters of 5-Bromodeoxyuridine</u>

Preparation of the mono- and di-amino acid esters of 5-bromouridine was carried out as described in Example 1C. A reactant ratio of 1:1 benzotriazole ester-to-5-bromodeoxyuridine was used to produce a mixture of both the mono- and di-esters; a reactant ratio of 2.5:1 benzotriazole-to-5-bromodeoxyuridine was utilized to favor formation of the di-ester conjugate. The BrdU mono esters were found to be separable from the diesters by column chromatography utilizing a stepwise eluent gradient and silica gel adsorbent. For syntheses employing reaction conditions favoring formation of the corresponding 2',5'-diesters of lysine, PheAla, Gly, Ala, and 6-aminocaproic acid respectively: 5-bromo-2'deoxy-5',3'-O,O-di-(2,6-diaminohexanoyl)uridine, 5-bromo-2'deoxy-5',3'-O,O-di-(2-amino-3-

phenylpropanoyl)uridine, 5-bromo-2'deoxy-5',3'-O,O-di-(aminoethanoyl)uridine, 5-bromo-2'deoxy-5',3'-O,O-di-(2-aminopropanoyl)uridine, 5-bromo-2'deoxy-5',3'-O,O-di-(6-aminohexanoyl)uridine, isolated yields were typically about 60%.

Example 2

Preparation of a Hydrophilic Polymer Linked with Phosphatidylethanolamine

A. Preparation of the Imidazole Carbamate of Polyethylene Glycol Methyl Ether 1900

9.5 g (5 mmol) of polyethylene glycol methyl ether 1900 was dissolved in 45 mL anhydrous benzene. 0.89 g (5.5 mmol) of pure carbonyl diimidazole was added. The reaction vessel was purged with nitrogen, sealed and heated in a sand bath at 75°C for 16 hours.

The reaction mixture was cooled and a clear solution formed at room temperature. The solution was diluted to a total volume of 50.0 mL with dry benzene and stored in the refrigerator as a 100 μl/mL stock solution. The activated polymer, the imidazole carbamate of PEG methyl ether 1900, was prepared for use in coupling to a vesicle-forming lipid, such as PE, as described below.

B. Preparation of the Phosphatidylethanolamine Carbamate of Polyethylene Glycol Methyl Ether 1900

10.0 ml (1 mmol) of the 100 mmol/ml stock solution from Example 2A was pipetted into a 10 mL pear-shaped flask. The solvent was removed under vacuum and 3.7 mL of a 100 mg/mL solution of egg phosphatidylethanolamine, PE, in chloroform (0.5 mmol) was added. The solvent was removed under vacuum. 2 mL of 1,1,2,2-tetrachloroethylene and 139 microliters (1.0 mmol) of triethylamine was added. The reaction vessel was sealed and maintained at 95°C for 6 hours. The extent of reaction was monitored by thin layer chromatography (TLC) on SiO_2 coated plates using a mixed solvent system eluent, butanone:acetic acid¦water, 8¦1¦1 (by volume). Visualization was carried out with iodine vapor. TLC revealed that most of the free phosphatidylethanolamine had reacted (Rf=0.68), and was replaced by a phosphorus-containing lipid spot with Rf=0.78-0.80.

The solvent from the reaction mixture was evaporated under vacuum. The residue was dissolved in 10 mL of methylene chloride and purified by column chromatography using Merck Kieselgel 60 (70-230 mesh) silica gel. The following solvent gradient was used to elute the products: 100 mL 100% CH_2Cl_2 (methylene chloride), 200 mL of 95/5 CH_2Cl_2/MeOH (methanol) containing 2% AcOH (acetic acid), 200 mL 90/10 CH_2Cl2/MeOH

containing 2% AcOH, 200 mL 85/15 CH_2Cl_2/MeOH containing 2% AcOH, and 200 mL 60/40 CH_2Cl_2/MeOH containing 2% AcOH.

50 mL portions of effluent were collected and assayed by TLC on silica-coated TLC plates, using I_2 visualization. A mixed solvent system containing chloroform:MeOH:water:conc. ammonium hydroxide, 130:70:8:0.5 by volume, was used to elute the samples. The majority of the phosphates were contained in fractions 11-14.

Fractions 11-14 were combined and dried to constant weight under high vacuum. A colorless wax product, phosphatidylethanolamine carbamate of polyethylene glycol methyl ethyl ether (669 mg, 52.6% yield), was recovered.

The product was characterized by proton NMR spectroscopy. A ^1H NMR spectrum of the product, dissolved in $CDCl_3$, contained resonances corresponding to the spectrum for egg PE, in addition to a strong singlet assigned to the methylene groups of the ethylene oxide chain at 3.4 ppm. Based on integration values, the ratio of methylene protons from the ethylene oxide chain to the terminal methyl group protons of the PE acyl groups was large enough to confirm a molecular weight of about 2000 for the polyethylene oxide portion of the desired product, polyethylene glycol conjugated phosphatidylethanolamine carbamate, M.W. = 2,654.

Example 3

A. Preparation of Rigid Long Circulating Liposomes: PEG-PE:HSPC:Chol Liposomes

A lipid matrix consisting of PEG-PE, HSPC and cholesterol (1:3:1 by weight) was dissolved in chloroform and the solvent was then removed by rotary evaporation to produce a thin film. The dried film was hydrated by addition of an ammonium sulfate buffer solution (250 mM, pH 5.5 ± 0.2) heated to 55 ± 5 C. The sample was maintained in a hot water bath, with mechanical shaking, for about 1 hour to ensure complete hydration of the film. Particle downsizing was accomplished by extrusion through polycarbonate filters (Szoka, 1980). The hydrated sample was extruded through polycarbonate membranes (Nucleopore, Pleasanton, CA) with defined pore sizes until the particle size was less than 100 nm (approximately 85 ± 5 nm).

An ammonium gradient was formed by carrying out consecutive dialysis exchanges of the extruded sample against a 10% sucrose solution at pH 5.8. The extruded sample underwent three one hour exchanges and one overnight exchange in a 1:20 by volume buffer solution. Final liposome samples with a weight ratio of 1:3:1 PEG-PE:HSPC:Chol at 100 umol/mL total lipid were isolated.

B. Preparation of Fluid Long Circulating Lipo-somes: PEG-PE:EPC IV 40:Chol liposomes

Liposomes with a weight ratio of 1:3:1 PEG-PE:EPC IV 40:Chol at 100 umol/mL total lipid were prepared as in Example 3A.

C. Preparation of Conventional Liposomes

Liposomes were prepared as described in Example 3A, except with a compositional ratio of PG:HSPC:cholesterol at 5:56:39 mol%.

Example 4

A. Loading of Nucleoside Analog-Amino Acid Ester Conjugates into Liposomes with an Ammonium Ion Gradient

The AZT-amino acid esters and BrdU-amino acid di-esters described in Examples 1C,D were each dissolved in 10% sucrose buffer to form stock solutions containing 5mg/mL ester. To 0.2 mL of the drug-containing stock solution was added 0.2 mL of the liposomal solutions from Examples 3A,3B. The resultant drug-liposomal mixture was then heated at 50° ± 5°C for 0.5 hour. The samples were cooled immediately in an ice bath and maintained in the ice bath for an additional 0.5-0.75 h prior to assaying for loading efficiency.

B. Loading Efficiency Assay for Nucleoside Analog-Amino Acid Ester Conjugates

The samples from Example 4A were analyzed to determine the amount of drug conjugate loaded into the liposomes. Prior to carrying out the analysis, a Sephadex G-50 gel filtration column (0.7 × 20 cm) was equilibrated with 0.9% saline. Drug-containing liposomal samples (150 uL) were loaded onto the column and the samples were eluted with saline. 300 uL fractions (10 drops) were collected and 900 uL of a 1:5 chloroform:methanol solvent system was added to each fraction.

The concentrations of loaded drug were determined spectrophotometrically based on their molar extinction coefficients. Quartz cuvettes were used for all samples. A wavelength of 266 nm was used for detection of AZT derivatives; a wavelength of 278 was used for derivatives of BrdU. Recoveries from the column were calculated based upon the absorbance of samples containing 10 uL of the loaded drug-liposome composition in 290 uL of saline and 900 uL of the chloroform-methanol solvent. Drug loading data is summarized in Tables I and II.

Table I.

Ester	Lipid Formulation	Percent Recovery	Percent Loading*
AZT-Lys	1:3:1 PEG-PE:HSPC:Chol	96	25
AZT-Lys	1:3:1 PEG-PE:EPC IV 40:Chol	77	29
AZT-Phe	1:3:1 PEG-PE:HSPC:Chol	99	62
AZT-Phe	1:3:1 PEG-PE:EPC IV 40:Chol	84	58
AZT-B Ala	1:3:1 PEG-PE:HSPC:Chol	~100	54
AZT-B Ala	1:3:1 PEG-PE:EPC IV 40:Chol	106	60
AZT-cap acid	1:3:1 PEG-PE:HSPC:Chol	84	64
AZT-cap acid	1:3:1 PEG-PE:EPC IV 40:Chol	88	60

*One hundred percent loading = 1.0 mg drug-conjugate

Table II.

Ester	Lipid Formulation	Percent Recovery	Percent Loading*
BrdU-Phe	1:3:1 PEG-PE:HSPC:Chol	137(?)	50
BrdU-Phe	1:3:1 PEG-PE:EPC IV 40:Chol	81	60
BrdU-Gly	1:3:1 PEG-PE:HSPC:Chol	95	20
BrdU-Gly	1:3:1 PEG-PE:EPC IV 40:Chol	93	26
BrdU-B Ala	1:3:1 PEG-PE:HSPC:Chol	114	9
BrdU-B Ala	1:3:1 PEG-PE:EPC IV 40:Chol	112	9
BrdU-cap acid	1:3:1 PEG-PE:HSPC:Chol	-	15
BrdU-cap acid	1:3:1 PEG-PE:EPC IV 40:Chol	85	14

*One hundred percent loading = 1.0 mg drug-conjugate

Control studies carried out on the unmodified parent nucleosides, AZT and BrdU, resulting in drug loading levels ranging from 1-8%. In comparison, the drug loading levels for the AZT-amino acid conjugates prepared in accordance with the present invention ranged from about 25-64%, while the loading levels for the BrdU-amino acid conjugates ranged from about 10-60%.

As shown above, the compound conjugates of the present invention exhibit enhanced liposome loading levels over those of the unmodified parent compounds.

Preliminary experiments carried out with the corresponding BrdU mono amino acid-esters resulted in loading levels similar to those in Table II.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the intended scope of the invention.

Example 5

A. Preparation of 5',3'-(5-iododeoxyuridine)diglutarate

The following reagents were added sequentially to a reaction flask: dimethylformamide (25 ml), iododeoxyuridine (2.87 g, 8.12 mmol), glutaryl anhydride (2.0 g, 17.86 mmol), and 4-DMAP (0.02 g, 0.18 mmol). The reaction mixture was stirred for 91 h at room temperature. The progress of the reaction was monitored by thin layer chromatography (eluent: 95/5 ethanol/ammonium hydroxide) using either UV or iodine visualization. During the progress of the reaction, the disappearance of starting material, iododeoxyuridine, was noted (R_f=0.60), and replaced by the appearance of two spots exhibiting lower R_f values, which were identified as the mono (R_f=0.20) and diglutarate (R_f=0.07) conjugates. The mixture of products was dried under high vacuum to yield an orange oil.

B. Preparation of 5',3'-(5-iododeoxyuridine) disuccinate

The following reactants were added sequentially to a flask: dimethylformamide (25 ml), iododeoxyuridine (3.00 g, 8.47 mmol), succinic anhydride (1.87 g, 18.64 mmol), and 4-DMAP (0.02 g, 0.19 mmol), and the resulting mixture was stirred for 72 h at room temperature. The reaction was monitored by thin layer chromatography (eluent: chloroform/ethanol/ammonium hydroxide, 2/19/1 by volume) using UV visualization. During the course of the reaction, disappearance of the starting material, iododeoxyuridine, was noted. The disappearance of starting material was accompanied by the appearance of two spots with lower R_f values, assigned as the mono and disuccinate products. The reaction was complete after 72 h, as indicated by the presence of a single low R_f spot by TLC, corresponding to complete conversion to the disuccinate product (R_f=0.17). Solvent was evaporated *en vacuo* to yield an oil (6.64 g). To the oil was added 125 ml of distilled water, resulting in a turbid solution. The solution was filtered, under vacuum, to yield a gummy precipitate. The precipitate was then dissolved in a minimum amount of methanol, to which was added a minimum amount of water. The pH of the solution was raised by addition of 1.0 N NaOH to form the corresponding disodium salt. The resulting solution was placed in

an isopropyl alcohol/dry ice bath and subsequently lyophilized to yield 1.18 g white powder.

IT IS CLAIMED:

1. A liposomal composition for use in administering to a subject, a compound having a derivatizable hydroxyl group, where said compound is substantially uncharged at neutral pH, comprising:

an external bulk-phase medium,

suspended in the medium, liposomes having an inside/outside pH gradient between an internal aqueous phase and the external bulk-phase medium, and

encapsulated within the liposomes, a compound conjugate composed of the compound to be administered and an adduct conjugated to the compound's hydroxyl group by an ester linkage,

said adduct being effective to maintain the compound conjugate in an ionized liposome-encapsulated form in response to said pH gradient.

2. The composition of claim 1, wherein the inside/outside pH gradient is a lower inside/higher outside pH gradient.

3. The composition of claim 2, wherein said adduct is an amino acid coupled to the compound through an ester linkage formed between the compound's hydroxyl group and the α carboxyl group of the amino acid.

4. The composition of claim 3, for use in treating tumor cells localized in a tissue, by irradiating such tissue in the presence of a tumor-targeted radiosensitizer compound, wherein the compound to be administered is a radiosensitizer compound selected from the group consisting of a halogenated deoxyuridine, a nitroimidazole, an anthraquinone, a 2-nitrofuran, a benzoquinone, and a naphthoquinone.

5. The composition of claim 3, wherein the compound to be administered is a nucleoside analog selected from the group consisting of 5'-chlorodeoxyuridine, 5'-bromodeoxyuridine, 5'-iododeoxyuridine, and 5'-fluorodeoxyuridine.

6. The composition of claim 2, wherein said lower inside/higher outside pH gradient is due to a higher inside/lower outside ammonium ion gradient.

7. The composition of claim 6, wherein said gradient is produced by an ammonium salt having a counterion which is effective to decrease the solubility of the compound conjugate in said internal aqueous phase.

8. The composition of claim 1, wherein the liposomes are composed predominantly of lipid components having phase transition temperatures above about 37°C.

9. The composition of claim 1, wherein the liposomes are coated with a hydrophilic polymer effective to extend the circulation time of the administered liposomes severalfold over that in the absence of such hydrophilic polymer.

10. The composition of claim 9, wherein the liposomes have sizes predominantly in the range between 0.07 and 0.12 μm.

11. The composition of claim 1, wherein the inside/outside pH gradient is a higher inside/lower outside pH gradient.

12. The composition of claim 11, wherein said adduct is a cyclic anhydride or a dicarboxylic acid coupled to the compound to form a carboxy-containing compound conjugate.

13. The composition of claim 12, for use in treating tumor cells localized in a tissue, by irradiating such tissue in the presence of a tumor-targeted radiosensitizer compound, wherein the compound to be administered is a radiosensitizer compound selected from the group consisting of a halogenated deoxyuridine, a nitroimidazole, an anthraquinone, a 2-nitrofuran, a benzoquinone, and a naphthoquinone.

14. The composition of claim 12, wherein the compound to be administered is a nucleoside analog selected from the group consisting of 5'-chlorodeoxyuridine, 5'-bromodeoxyuridine, 5'-iododeoxyuridine, and 5'-fluorodeoxyuridine.

15. The composition of claim 11, where the pH of the internal aqueous phase is at least 2 units greater than the pK_a of the carboxy group of the compound conjugate.

16. The composition of claim 11, wherein said higher inside/lower outside pH gradient is due to a higher inside/lower outside bicarbonate ion gradient.

17. A drug-delivery combination for use in administering to a subject, a compound having a derivatizable hydroxyl group, where said compound is substantially uncharged at neutral pH, comprising:

a compound-conjugate composed of the compound to be administered and an adduct conjugated to the hydroxyl group of such compound by an ester linkage, and

liposomes, which, when suspended in an aqueous medium having a selected pH, possess an internal aqueous phase and an inside/outside pH gradient between the internal phase and the aqueous medium,

where said adduct is effective to cause net uptake of the compound into the liposomes in response to said pH gradient.

18. The combination of claim 17, wherein the inside/outside pH gradient is a lower inside/higher outside pH gradient.

19. The combination of claim 18, wherein said lower inside/higher outside pH gradient is due to a higher inside/lower outside ammonium ion gradient.

20. The combination of claim 19, wherein said gradient is produced by an ammonium salt having a counterion which is effective to decrease the solubility of the compound conjugate in said internal aqueous phase.

21. The combination of claim 20, wherein the compound is selected from the group consisting of 5'-chlorodeoxyuridine, 5'-bromodeoxyuridine, 5'-iododeoxyuridine, and 5'-fluorodeoxyuridine, and where said adduct is an amino acid coupled to the compound through an ester linkage formed between the compound's hydroxyl group and the α carboxyl group of the amino acid.

22. The combination of claim 17, wherein the inside/outside pH gradient is a higher inside/lower outside pH gradient.

23. The combination of claim 22, wherein said adduct is a cyclic anhydride or a dicarboxylic acid coupled to the compound to form a carboxy-containing compound conjugate.

24. The combination of claim 23, wherein the compound is selected from the group consisting of 5'-chlorodeoxyuridine, 5'-bromodeoxyuridine, 5'-iododeoxyuridine, and 5'-fluorodeoxyuridine.

25. A method of preparing a suspension of liposomes for use in delivering a compound having a derivatizable hydroxyl group, where said compound is substantially uncharged at neutral pH, comprising:

adding to an aqueous suspension containing (i) a bulk phase medium, and (ii) suspended in the bulk phase medium, liposomes having an internal aqueous phase, and an inside/outside pH gradient between said internal aqueous phase and the bulk-phase medium, a derivatized compound composed of the compound to be delivered, and an adduct attached to the compound's hydroxyl group through an ester linkage, and

incubating the compound and liposomes under conditions effective to allow passage of the compound from the external medium into the liposomes,

wherein the presence of said adduct is effective to produce net uptake of the compound conjugate into the liposomes in response to said pH gradient.

26. The method of claim 25, wherein said inside/outside pH gradient is a lower inside/higher outside pH gradient.

27. The method of claim 26, wherein said lower inside/higher outside pH gradient is due to a higher inside/lower outside ammonium ion gradient.

28. The method of claim 27, wherein said gradient is produced by an ammonium salt having a counterion which is effective to decrease the solubility of the compound conjugate in said internal aqueous phase.

29. The method of claim 25, wherein the liposomes in the suspension are formed predominantly of lipids having phase transition temperatures above about 37°C, and said incubating is carried out at a temperature substantially above the phase transition temperatures of the liposome-forming lipids.

30. The method of claim 26, wherein said pH gradient is produced by selecting an internal aqueous phase pH between about 4.5 and 7.5 and at least 2 pH units lower than the pH of the external bulk phase medium.

31. The method of claim 25, wherein the inside/outside pH gradient is a higher inside/lower outside pH gradient.

32. The method of claim 32, wherein said higher inside/lower outside pH gradient is due to a higher inside/lower outside bicarbonate ion gradient.

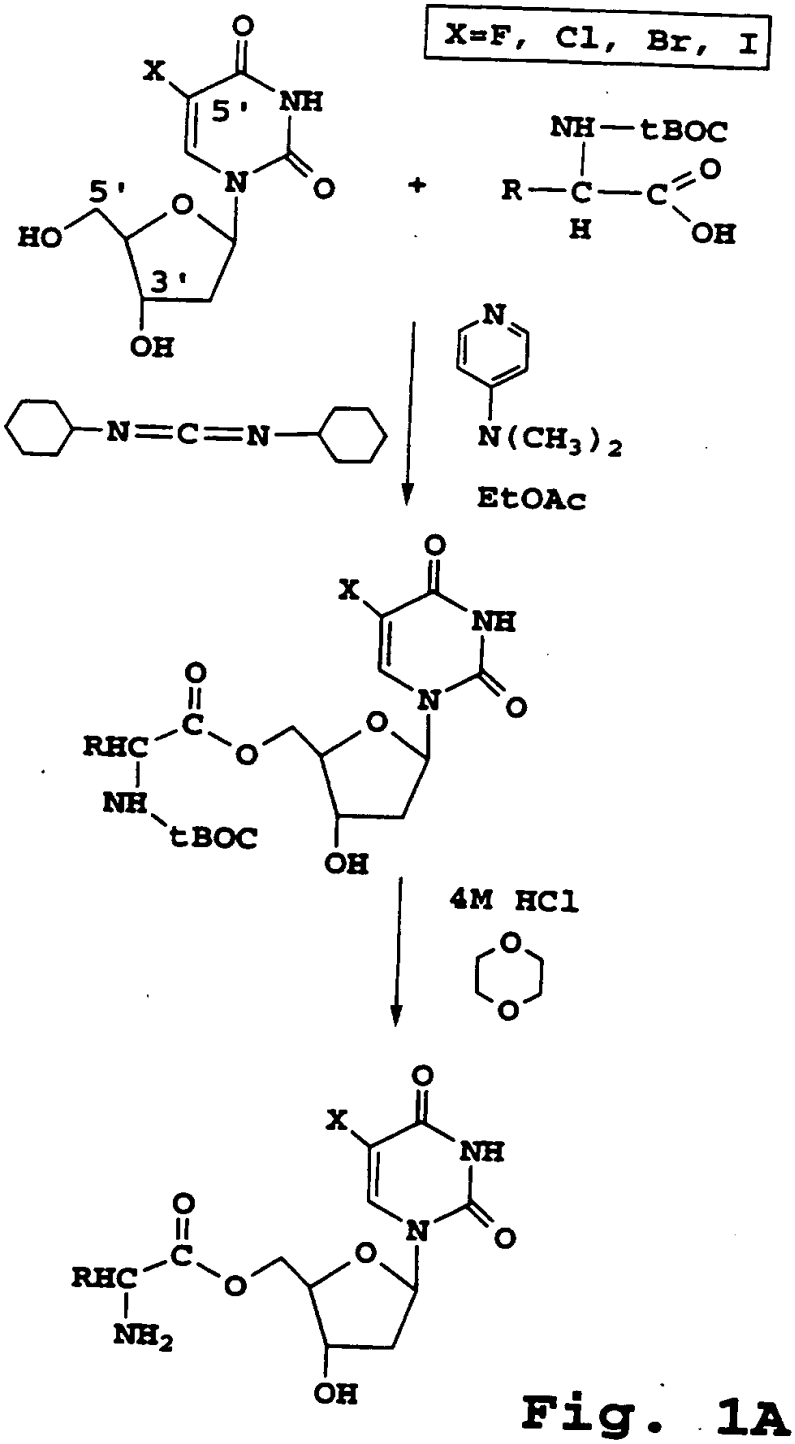
X=F, Cl, Br, I
NH—tBOC
R—C—C
H
OH
HO
5'
3'
OH
N=C=N
N(CH3)2
EtOAc
RHC
NH
tBOC
4M HCl
NH2

Fig. 1A

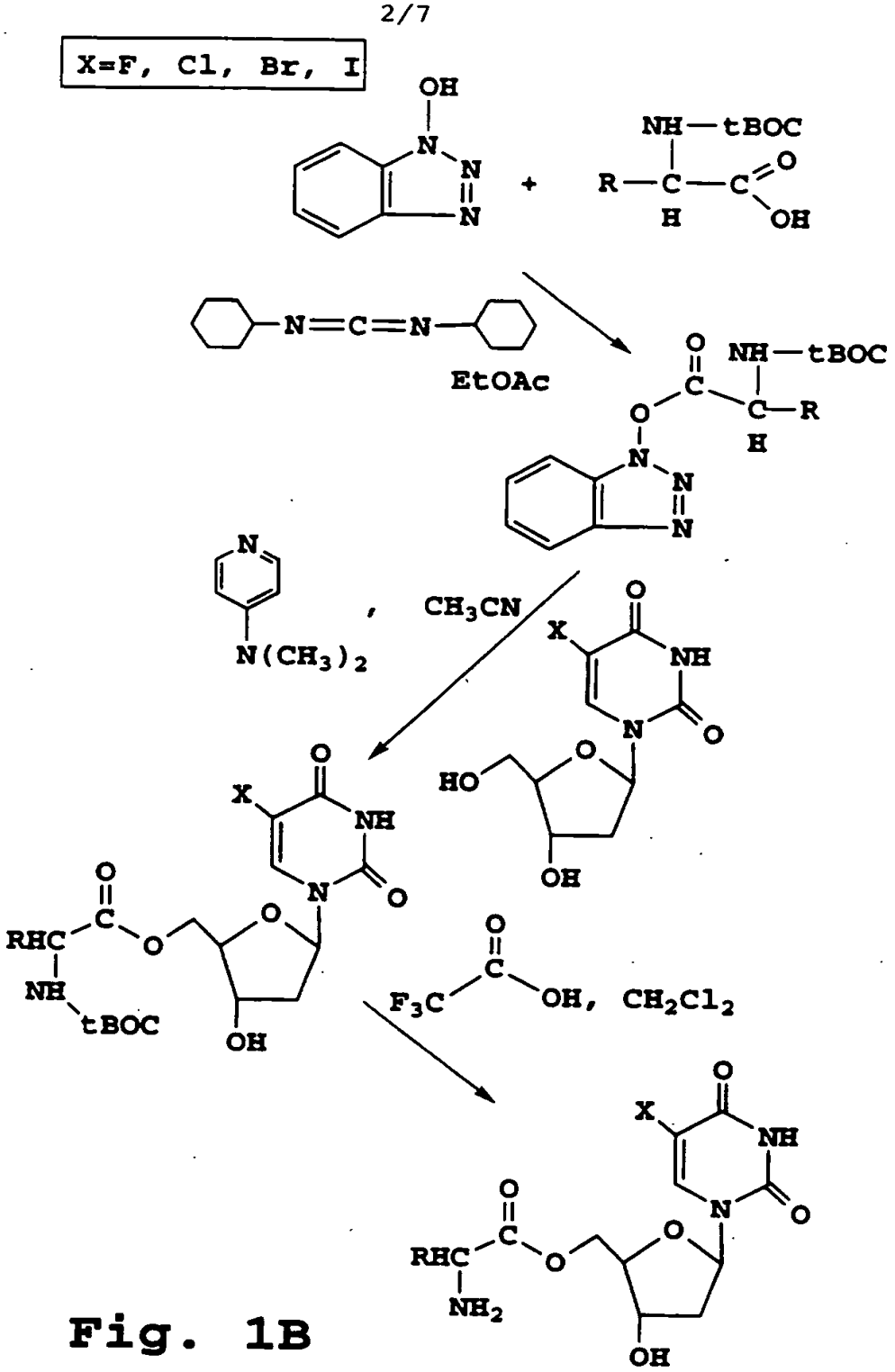
X=F, Cl, Br, I
OH
N
N
N
+
NH—tBOC
R—C—C=O
H
OH
N=C=N
EtOAc
O
NH—tBOC
C
O
C—R
H
N
N
N
N
N(CH3)2
,
CH3CN
X
NH
N
O
O
HO
O
OH
X
NH
N
O
O
RHC
O
O
NH
tBOC
OH
F3C
C
O
OH, CH2Cl2
X
NH
N
O
O
RHC
O
O
NH2
OH

Fig. 1B

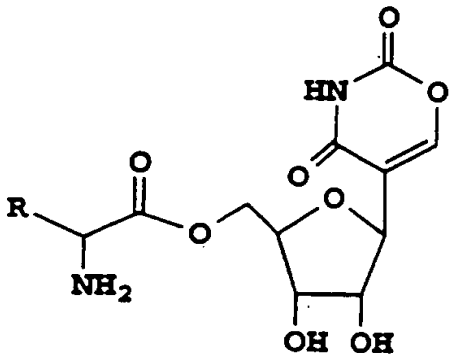

Fig. 2A

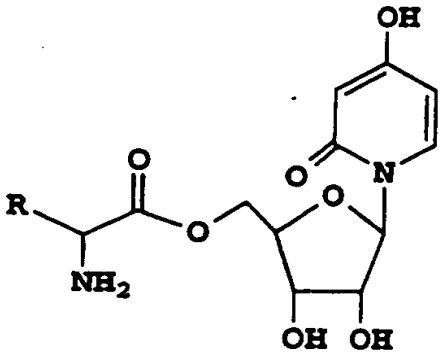

Fig. 2B

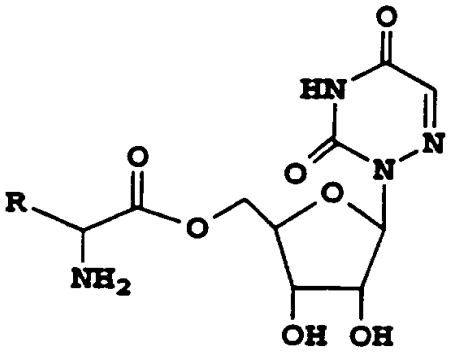

Fig. 2C

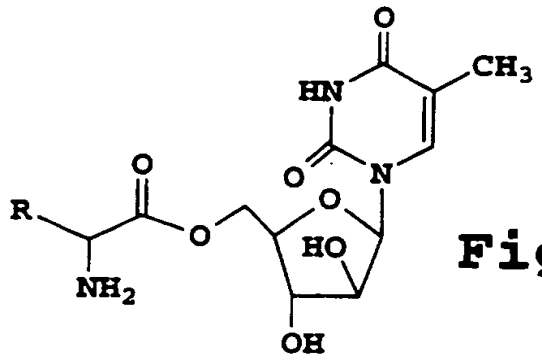

Fig. 2D

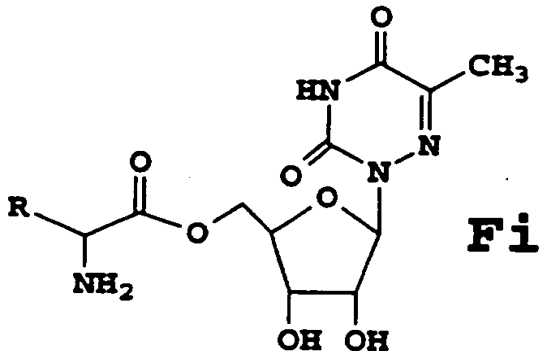

Fig. 2E

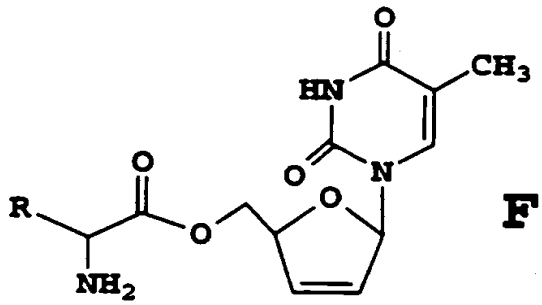

Fig. 2F

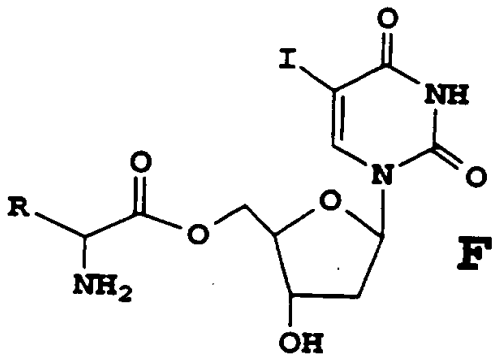

Fig. 3A

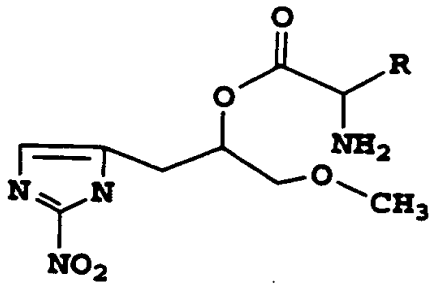

Fig. 3B

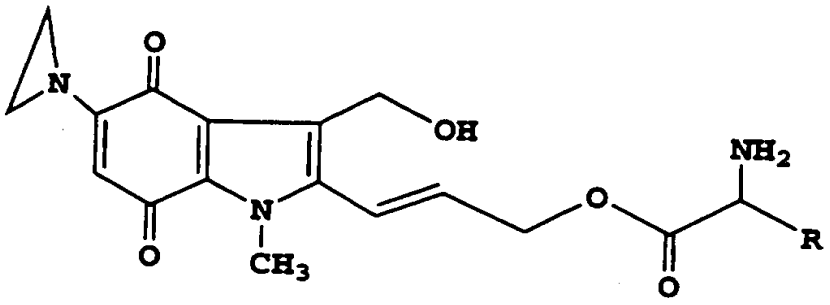

Fig. 3C

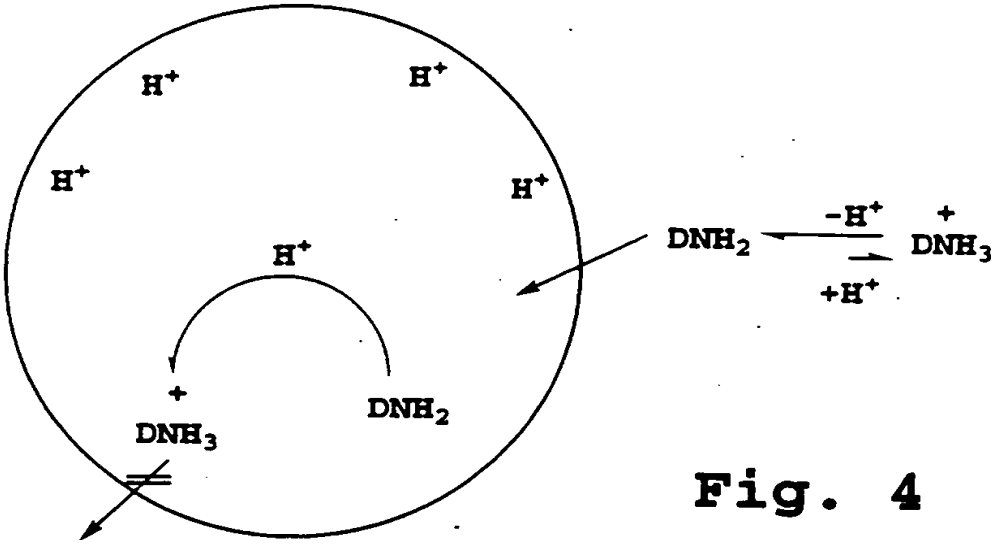

Fig. 4

D = Drug conjugate

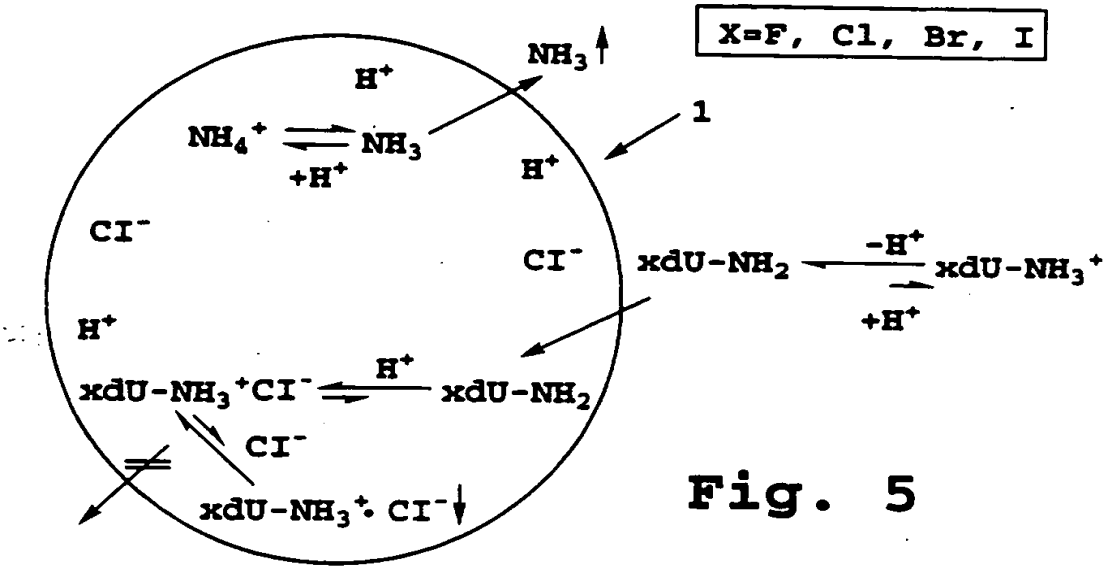

Fig. 5

xdU=halodeoxyuridine conjugate

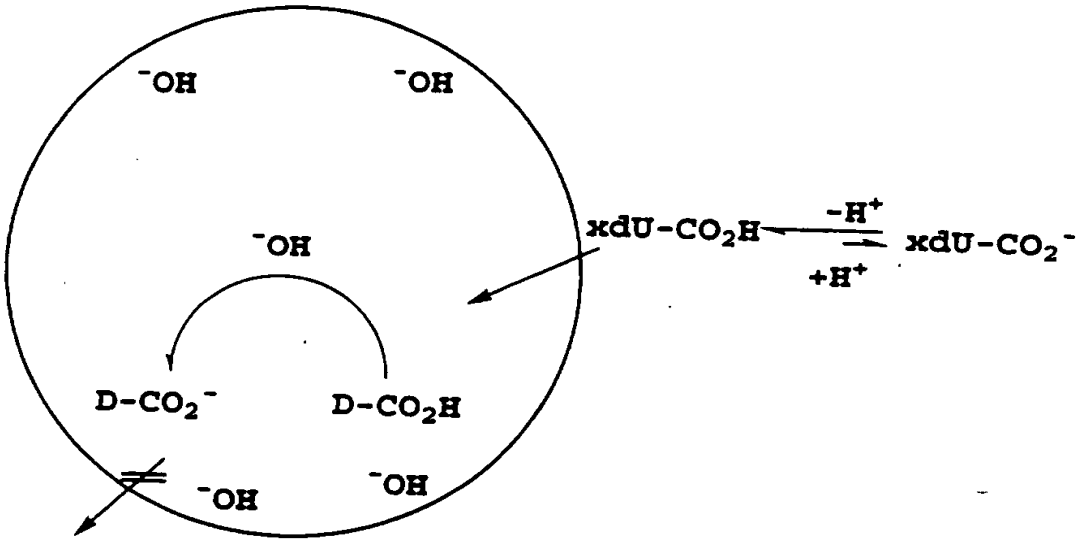

Fig. 6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/02005

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 361 894 (YISSUM RES. DEVELOP. COMP. HEBREW UNIVERS. JERUSALEM) 4 April 1990 see the whole document & US,A,5 192 549 cited in the application	1-32
A	WO,A,92 02244 (THE LIPOSOME COMPANY, INC.) 20 February 1992 see the whole document	1-32

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
3 July 1996	15.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Benz, K

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/02005

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-361894	04-04-90	AT-T-	113830	15-11-94
		CA-A-	1335565	16-05-95
		DE-D-	68919321	15-12-94
		DE-T-	68919321	24-05-95
		ES-T-	2067551	01-04-95
		US-A-	5192549	09-03-93
		US-A-	5316771	31-05-94
WO-A-9202244	20-02-92	AT-T-	138803	15-06-96
		AU-B-	660288	22-06-95
		AU-B-	8436591	02-03-92
		CA-A-	2087965	01-02-92
		EP-A-	0555229	18-08-93
		JP-T-	6501246	10-02-94
		US-A-	5380531	10-01-95